The role of clinical criteria, genetic and epigenetic alterations in Lynch-syndrome diagnosis

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Lynch syndrome (hereditary nonpolyposis colorectal cancer, HNPCC) represents 1-3% of all diagnosed colorectal cancers (CRCs). This study aimed to evaluate the benefit of clinical criteria and several molecular assays for diagnosis of this syndrome. We examined tumors of 104 unrelated clinically characterized colorectal cancer patients for causal mismatch repair (MMR) deficiency by several methods: microsatellite instability (MSI) and loss of heterozygosity (LOH) presence, MMR protein absence, hypermethylation of *MLH1* promoter and germline mutation presence. Twenty-five (24%) patients developed CRCs with a high level of MSI (MSI-H). Almost all (96%) had at least one affected relative, while this simple criterion was satisfied in only 22% (17/79) of individuals with low level MSI or stable cancers (MSI-L, MSS). Using strict Amsterdam criteria, the relative proportion of complying individuals in both sets of patients (MSI-H vs. MSI-L and MSS) decreased to 68% and 9%, respectively. The right-sided tumors were located in 54% of MSI-H persons when compared to 14% of cancers found in MSI-L or MSS patients. In 16 MSI positive patients with identified germline mutation by DNA sequencing, the gene localization of mutation could be indicated beforehand by LOH and/or immunohistochemistry (IHC) in four (25%) and 14 cases (88%), respectively. The IHC findings in MSI-H cancers with methylation in distal or both regions of *MLH1* promoter have not confirmed the epigenetic silencing of the *MLH1* gene. None of the patients with MSI-L or MSS tumors was a carrier of the *MLH1* del616 mutation, despite seven of them meeting Amsterdam criteria. The effective screening algorithm of Lynch-syndrome-suspected patients consists of evaluation of Bethesda or Revised Bethesda Guidelines fulfilling simultaneous MSI, LOH and IHC analyses before DNA sequencing. Variable methylation "background" in *MLH1* promoter does not affect gene silencing and its role in Lynch-syndrome tumorigenesis is insignificant.

Keywords: Lynch-syndrome diagnosis, clinical criteria, microsatellite instability, loss of heterozygosity, MMR protein expression, MLH1 methylation.

Lynch syndrome (hereditary nonpolyposis colorectal cancer, HNPCC) is the most common autosomal dominant disease that predisposes patients to cancer, mainly colorectal and endometrial adenocarcinomas, less frequently tumors of stomach, small bowel, hepatobiliary tract, upper urologic tract, ovaries and brain [1]. In approximately 89% of typical Lynch-syndrome families, the inherited mutations are located in DNA mismatch repair genes *MLH1* or *MSH2*, less frequently in *MSH6, MLH3, PMS2* or *PMS1* genes [2]. The carriers of germline mutations are at 70% lifetime risk of developing colorectal or extracolonic cancer [3].

Clinical characters and cancer family history are critical data for identification of families suffering from Lynch syndrome. The index patient for germline mutation screening has been selected by using various sets of criteria: the more stringent are the Amsterdam criteria I (AC I) [4] and the Amsterdam criteria II (AC II) including extracolonic cancers [5] or the modified, less strict criteria - Bethesda Guidelines (BG 1-7) [6] or Revised Bethesda Guidelines (RBG 1-5), which have been lately updated by The International Collaborative Group on Hereditary NonPolyposis Colorectal Cancer (ICG-HNPCC) [7]. The majority (85-90%) of tumors in Lynch-syndrome patients display microsatellite instability (MSI) as a result of a DNA mismatch repair (MMR) deficiency [8]. This phenomenon is manifested by small insertions or deletions in repetitive units (about 1-6 motifs), which are commonly found in intron sequences, but also in exons of many cancer-related genes. Microsatellites located near or

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inside the relevant genes can be useful markers also for evaluation of loss of heterozygosity (LOH) that is frequently observed in colorectal cancers (CRCs). LOH at *MLH1* and *MSH2* loci occurs in both hereditary and sporadic CRCs with high level of MSI (MSI-H) [9-11]. The loss of wild-type allele indicates that the gene is affected by germline alteration. In most tumors, the significant indicator of MMR defect is the loss of MMR protein(s), as a result of degradation.

The diagnosis of Lynch-syndrome patients is costly and time consuming. Currently, there is an urgent need for an economical, sensitive, and specific testing strategy of the disease. A cost-effective strategy has been evaluated using different combinations of clinical criteria (AC and BG simultaneously, or BG alone), and MSI testing, and *MSH2* and *MLH1* sequencing [12-13]. Several authors have preferred the immunohistochemical evaluation (IHC) of MLH1, MSH2, MSH6 and PMS2 protein expressions instead of MSI testing because of the low cost approximately one third [14] and high sensitivity (92%) as well as specificity (100%) [15]. In several studies both assays, MSI and IHC, were considered to be effective pre-screening methods before germline mutation scanning by genomic sequencing of MMR genes [16-18]. At least two serious complications of this diagnostic procedure were uncovered. First, the pathogenic large genomic rearrangements representing up to 27% of all mutations [19] are not recognized by DNA sequencing. Second, the epigenetic inactivation of *MLH1* gene by promoter hypermethylation resulted in MSI-H sporadic CRCs with loss of *MLH1* expression [20, 21] and infrequently in Lynch-syndrome patients as the "second hit" [22, 23] is omitted.

Two extensive CpG islands in the *MLH1* promoter sequence were detected in regions from -778 bp to -458 bp and from -367 bp to -201 bp, respectively. Several assays, including methylation-sensitive enzyme digestion, combined-bisulfite restriction analysis (COBRA), and methylation-specific PCR (MSP), have been used for examining DNA methylation in several CpG sites distributed in various parts of the *MLH1* promoter. Due to variable results obtained, the promoter sequences that are responsible for the *MLH1* gene silencing remain unknown. However, several cell lines and CRCs exhibit limited methylation of promoter, determined by MSP, which correlated well with inhibition of MLH1 protein expression [24-26].

Lynch-syndrome diagnosis includes the clinical indicators, family history, and molecular-genetic analyses. However, accuracy in distinguishing hereditary tumors from sporadic cases requires that several facts should be considered. First, approximately 15% of sporadic CRCs showed MSI, and some of them met BG or RBG clinical criteria [27]. Second, rectal cancers are rarely MSI positive in multiple CRC families [28]. Third, is that in sporadic CRCs, the biallelic or hemiallelic hypermethylation of the promoter region of the wild-type *MLH1* allele frequently results in the MSI phenotype [29, 30]. Fourth, several Lynch-syndrome patients developed cancers with low or atypical MSI status, where the del616 germline mutation of the *MLH1* gene was identified [31]. At last, in carriers of pathogenic missense MMR mutations, expression of the relevant protein is generally normal [32].

In the present study, we examine a group of suspected Lynch-syndrome patients using various screening methods, including MSI, LOH, IHC, MSP and genomic sequencing. The aim of this study is to evaluate the benefit of different clinical criteria and molecular assays for effective diagnosis.

Material and methods

Patients and samples. In total, 104 unrelated CRC patients were selected and investigated. The clinical data and family history of patients were collected. Four sets of international clinical criteria for Lynch syndrome were used for comparison with molecular-genetic testing: Amsterdam criteria I and II (AC I, AC II), Bethesda Guidelines (BG 1-7) and Revised Bethesda Guidelines (RBG 1-5) (Table 1). DNA was extracted from matched normal and tumor samples (peripheral blood, fresh frozen tissues or paraffin embedded tissue sections) of each patient. Genomic DNA isolation from blood was performed as described previously [33]. DNA from fresh mechanically homogenized tissues was isolated by standard phenol-chloroform-butanol extraction and ethanol precipitation [34]. The sections of archival tissue with minimally 70% of malignant cells were deparaffinized by xylene and ethanol rinsing. The high molecular weight DNA was extracted and preamplified by whole genome amplification (WGA) to decrease DNA degradation [35]. The modified I-PEP PCR was performed in a 100 µl reaction mixture, containing 1.6 µM totally degenerated PCR primers 15 nucleotides long, 2.5 mM $MgCl_2$, 200 mM dNTP, 0.05 mg/1ml gelatine (final concentration), 7 U of a mix of Taq polymerase and proofreading Pwo polymerase (Expand High Fidelity PCR System, Roche, Mannheim, Germany), and 1.5 µg of archival substrate DNA in 1x buffer No.3. After a hot start at 94°C for 3 min, the samples were processed through 50 cycles; 94°C for 1 min, 37°C for 2 min, ramping step of 0.1°C/sec to 55°C, 55°C for 4 min and 68°C for 30 sec. The amplification was completed with 10 min long extension at 72°C. The PCR products were stored at 4°C until further processing.

MSI analyses. The evaluation of MSI status was performed on matched DNA samples (normal / tumor) of 54 CRC patients by isotopic-labelling analyses using the panel of markers as described previously [34]. The samples of the 50 remaining patients were analyzed by a high-resolution fluorescent-labelling method [36] using 5-10 highly polymorphic MSI markers: BAT26, BAT40, BATRII, BAX, D2S123, D3S1611, D17S250, D5S346, D18S34 and MYCL1 [37-42]. Forward primer for each MSI marker was labelled on the 5'-end by fluorescent dye (FAM, HEX or NED). PCRs were carried out in a 30 µl reaction mixture, containing 100 ng purified DNA (or aliquot 10 µl of I-PEP PCR from archival DNA), 0.33 µM of each forward and reverse primer, 200 µM dNTP, 1.5 mM $MgCl_2$, 1x DynaZyme EXT buffer and 1 U DynaZyme

Table 1. Clinical criteria for Lynch-syndrome-suspected patients

Name	Criteria
Amsterdam criteria I (AC I)	At least three relatives with CRC. All of following criteria should be met : One of these is first-degree relative of the other two At least two successive generations are affected At least one affected person is younger than 50 years Familial adenomatous polyposis is excluded
Amsterdam criteria II (AC II)	At least three relatives with an HNPCC-associated cancer (CRC, endometrium, small bowel, ureter, or renal pelvis). All of Amsterdam criteria I should be met.
Bethesda Guidelines (BG)	Individuals with cancer in families that fulfill the Amsterdam criteria Individuals with two HNPCC-related cancers, including synchronous or metachronous CRCs or associated extracolonic cancers (endometrial, ovarian, gastric, hepatobiliary or small bowel or transitional cell carcinoma of renal pelvis or ureter) Individuals with CRC and a first degree relative with CRC and/or HNPCC-related extracolonic cancer and/or colorectal adenoma; one of cancer diagnosed at age < 45 years and the adenoma diagnosed at age < 40 years Individuals with CRC or endometrial cancer diagnosed at age < 45 years Individuals with right-sided CRC with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed at age < 45 years Individuals with 'signet ring cells" type CRC diagnosed at age < 45 years (> 50% signet ring cells) Individuals with colorectal adenomas, diagnosed at age < 40 years
Revised Bethesda Guidelines (RBG)	The patient is younger than age 50 The patient has multiple HNPCC-associated tumors in the colon or in other areas known to be caused by the same mutation, either at the some time or occurring over a period of time A patient younger than age 60 has colorectal cancer that has microscopic characteristics that are often indicative of MSI A patient has one or more first-degree relatives who had an HNPCC-related tumor at the age 50 or younger A patient has two or more first- or second-degree relatives who had an HNPCC-related tumor at any age

EXT DNA Polymerase (FINNZYMES, Espoo, Finland) in final concentration. The PCR program consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 55-64°C for 30 sec, and 72°C for 1 min, and final extension at 72°C for 10 min. The amplification was finished with 10 min at 15°C and PCR products were stored at 4°C. The mixture of 1 µl PCR product dilution and 0.5 µl of ROX size standard GeneScan-500 in 15 µl formamide were denatured and electrophoretically separated in Performance Optimized Polymer-4 on an ABI PRISM 310 Genetic Analyzer. Collected data were evaluated by GeneScan software (Applied Biosystems, Foster City, CA, USA). The MSI marker was considered unstable when some extra peaks were present in tumor that differed from corresponding normal tissue. Tumors were classified as MSI-H if at least 30% of markers manifested an instability, as MSI-L (low level of MSI), if less than 30% positive MSI markers were identified, and as MSS (stable) for tumors without any MSI.

LOH analyses. LOH in tumors was identified by using two triplex PCR of the *MSH2* region (D2S391, D2S123, D2S378) and the *MLH1* region (D3S1561, D3S1611, D3S3685). The primer sequences are available in the UniSTS database [39]. PCRs were carried out in a 30 µl reaction mixture, which contained 100 ng of purified DNA, 1x QIAGEN Multiplex PCR Master Mix and 0.2 µM of each primer. PCR program was performed as described in the MSI cycling protocol, with annealing temperatures of 58°C and 60°C for the *MSH2* and *MLH1* gene regions, respectively [43]. Only clearly estimated heterozygous loci without novel alleles in tumor DNA were deemed informative. The sample was classified as LOH positive, when tumor DNA decreased, or when there was an absence of peaks in one allele of analyzed MSI marker in contrast with matched normal DNA. Mathematical calculation of LOH was performed according to the following formula:

$$\text{LOH} = \frac{(\text{height of normal allele two}) / (\text{height of normal allele one})}{(\text{height of tumor allele two}) / (\text{height of tumor allele one})}$$

LOH is positive if the value is ≤ 0.5 or ≥1.5 [44].

IHC analyses. Expressions of MLH1, MSH2, and in several cases, also MSH6 and PMS2 proteins were analyzed by IHC as described previously [45]. The primary monoclonal antibodies were applied as follows: 1.2 µg/ml of anti-hMLH1 (Ab13271A, Pharmingen, Basel, Switzerland) for 1 hour in room temperature (RT); 1 µg/ml of anti-hMSH2 (Ab NA26, Oncogene Research, Darmstadt, Germany) for 24 hours at 4°C; 4 µg/ml of anti-hMSH6 (Ab G70220, Transduction Laboratories, Lexington, United Kingdom) for 2 hours at RT and 3 µg/ml of anti-hPMS2 (Ab 65861A, Pharmingen, Basel, Switzerland) for 24 hours at 4°C. The inhibition of protein expression in tumor tissue was classified as positive if the staining was very weak or none in contrast with normal stained colonocytes and stroma cells in the samples of the same patient.

Hypermethylation of MLH1 promoter. Genomic DNA was modified with sodium bisulfite according to the protocol of the CpGenome™ Modification Kit (Chemicon International, Temecula, CA, USA) [46]. The principle of the assay is that unmethylated cytosines in CpG dinucleotides convert to uracils while 5-methylcytosines remain unaltered. For the modification procedure, 1 µg of genomic DNA (alternatively 2 µg of archival DNA) was used. CpG methylation status was ana-

lyzed by MSP in the A and B regions of *MLH1* promoter (from -716 bp to -602 bp and -248 bp to -178 bp from transcription start), respectively. MSP was performed in two separate PCRs using specific primers for unmethylated or methylated DNA [24, 26]. PCRs were carried out in a 30 µl reaction mixture, containing 100 ng of modified DNA (or 400 ng of archival DNA), 0.66 µM of each forward and reverse primer, 200 µM dNTP, 1.5 mM $MgCl_2$, 1x PCR buffer and 1.2 U HotStarTaq DNA polymerase (QIAGEN, Hilden, Germany) in final concentration. The PCR program consists of an initial denaturation at 95°C for 15 min, followed by 40 cycles at 94°C for 30 sec, 57°C (both regions) in testing of unmethylated DNA substrate or 62°C and 65°C (A and B, respectively) for methylated DNA substrate for 30 sec, and 72°C for 30 sec. The final extension was performed for 10 min at 72°C. The PCR products were estimated using 2.5% agarose gel electrophoresis with ethidium bromide staining. The samples of each patient were evaluated for the presence of PCR products in four variants: PCR for unmethylated and methylated substrates in both normal and tumor DNA.

DNA genomic sequencing. Mutation analyses were performed by direct genomic sequencing of *MLH1* and *MSH2* genes in most of the MSI-H patients as described previously [47]. Individuals with MSI-L or MSS tumors, which fulfilled at least one clinical criterium for Lynch syndrome were screened for del616 mutation in exon 16 of the *MLH1* gene.

Results

Clinical criteria in groups with different MSI status. The samples from 104 unrelated colorectal cancer patients were collected in the period from 1998 to 2005 from several hospitals of Slovakia. The patients were less than 50 years old with single, synchronous or metachronous carcinomas, and/or had relatives who suffered from Lynch-syndrome-associated tumors (carcinomas of colorectum, endometrium, stomach, renal pelvis). In several families, cancers with vague relationship to Lynch syndrome (cancers of breast, brain, prostate, liver, lung, leukaemia, skin, tongue, larynx, and melanoma) were recorded.

All patients were analyzed for the presence of MSI in their tumor tissues; 25 (24%) of the samples manifested MSI-H phenotype, 13 (12.5%) and 66 (63.5%) were MSI-L and MSS, respectively. MSI results of several patients (codes 1-4 and 27) were published in a recent study [34]. Clinical criteria, location and multiplicity of tumors in patients with different status of MSI are summarized in Table 2.

Out of 25 patients with MSI-H tumors, 24 had at least one relative who developed Lynch-syndrome-associated cancer. The Amsterdam criteria (AC I or AC II) met families of 17 patients. Of those four individuals younger than 45 years had one relative with any related cancer (BG 3), and three patients had one or more family members with stomach (RBG 4) or endometrial tumors (RBG 2,5) diagnosed before 50 years of age. One young woman developed cancer at 49 years (RBG 1). The right-sided MSI-H carcinomas (cecum, ascending colon, hepatic flexure, transverse colon) were found in 10 patients, and four patients manifested CRCs with at least one on the right side. Left colon carcinomas were located from splenic flexure to rectum in 11 individuals.

Of the 13 MSI-L patients, only four had at least one relative who suffered from related cancers (AC I or BG 3). The tumors were located mostly in the left colon. Out of 66 unrelated persons with stable carcinomas, 13 persons had cancer in their family history, in which six and seven of the cases fulfilled the strict criteria (AC I or AC II) and less stringent guidelines (BG 3, RBG 4 or RBG 5), respectively. One 44-year-old woman and three elder men developed metachronous carcinomas (BG 2). However, 17 other patients with early-onset tumors were diagnosed at an average age of 35 ± 6.5 years (BG 4). Almost all MSS tumors were located in the distal colon, except for eight individuals with right-sided carcinomas. The data from a selected group of 18 CRC patients with MSS phenotype are presented in Table 2.

Genetic and epigenetic analyses in MSI-H tumors. Cancers with MSI-H were analyzed for LOH by two triplexes of markers in surrounding sequences of *MSH2* and *MLH1* genes, which covered the regions of 10.9 Mbp in 2p chromosome and 6 Mbp in 3p chromosome, respectively. The evaluating system was designed according to LOH results performed in the group of 34 MSI-L or MSS tumors. Out of 14 tumors manifesting LOH in one of two regions, the complete absence of all three markers was evidenced in four cases, the other two samples manifested LOH in two markers of *MSH2* region (including D2S123), and in five carcinomas, the chromosomal loss in one or two markers of *MLH1* region with other uninformative ones was shown. Only in three cancers, a combination of negative results in D2S123 or D3S1611 and LOH in downstream markers was found. The results of LOH are summarized in Table 3. These data document that 79% of examined MSI-L or MSS tumors show extensive loss in the sequences of the surrounding *MSH2* and *MLH1* genes. Problematic evaluation of LOH in MSI-H cancers can be improved using at least one marker of triple set because of relatively high probability of larger chromosomal deletions covering also the sequences, where the uninformative markers are located. The example of two markers with LOH presence and one uninformative because of MSI in triplex PCR is presented in Figure 1. Effectiveness of LOH evaluation by triplex PCR in regions including *MSH2* or *MLH1* genes comparing with uniplex PCR (only D2S123 or D3S1611) increased from 0% to 8.7% and from 34.8% to 47.8%, respectively. LOH results of 23 MSI-H tumors are presented in Table 4. Two informative tumor samples were negative in *MSH2* region, from 11 informative cases in *MLH1* region, six samples (54.5%) were positive.

Immunohistochemical analyses of MSH2 and MLH1 proteins were performed in 20 MSI-H carcinomas, and nearly all samples were stained for MSH6 and PMS2 proteins. The absence of protein immunostaining in paraffin block sections of tumor tissue is caused by mutations in relevant genes, which

Table 2. Clinical criteria and tumor locations in CRC patients with different MSI status

Patient code	Age	MSI status[a]	Lynch syndrome criteria[b]	Colorectal tumor location
1.	46	H	AC I	3 synchronous, all colon ascendens
2.	34	H	RBG 4	cecum
3.	32	H	BG 3	sigmoid colon
4.	27	H	BG 3	cecum
5.	36	H	AC I	rectosigmoid
6.	35	H	AC I	colon ascendens
7.	43	H	RBG 4	splenic flexure
8.	43	H	AC I	colon ascendens
9.	36	H	BG 3	rectosigmoid
10.	40	H	AC I	colon transversum
11.	34	H	RBG 2,5	2 synchronous: cecum, colon transversum
12.	36	H	AC II	sigmoid colon
13.	52	H	AC I	colon ascendens (+ Ca endometrium)
14.	49	H	RBG 1	cecum
15.	36	H	AC I	colon ascendens
16.	30	H	AC I	rectosigmoid
17.	27	H	AC I	2 synchronous: colon ascendens, sigmoid colon
18.	29	H	BG 3	colon transversum
19.	32	H	AC II	3 metachronous: one colon ascendens
20.	42	H	AC I	colon descendens (+ Ca ovarii)
21.	52	H	AC I	colon ascendens (+ Ca endometrium)
22.	28	H	AC I	rectum
23.	35	H	AC I	colon descendens
24.	49	H	AC II	sigmoid colon
25.	36	H	AC I	splenic flexure
26.	34	L	BG 3	rectum
27.	50	L	RBG 1	rectosigmoid
28.	28	L	BG 4	rectosigmoid
29.	38	L	BG 4	rectum
30.	26	L	BG 4	sigmoid colon
31.	48	L	RBG 1	colon
32.	31	L	BG 3	rectum
33.	47	L	RBG 1	rectum
34.	33	L	BG 4	colon ascendens
35.	37	L	BG 4	rectum
36.	48	L	AC I	colon ascendens
37.	53	L	BG 2,3	2 synchronous: colon ascendens, cecum
38.	24	L	BG 4	sigmoid colon
39.	46	S	RBG 1	rectum
40.	49	S	BG 3	rectum
41.	36	S	BG 4	rectosigmoid
42.	33	S	BG 4	colon
43.	42	S	BG 4	sigmoid colon
44.	32	S	BG 4	rectosigmoid
45.	37	S	BG 4	colon ascendens
46.	69	S	AC II	rectum
47.	39	S	BG 3	sigmoid colon
48.	45	S	AC I	sigmoid colon
49.	22	S	BG 4	sigmoid colon
50.	42	S	AC I	rectum
51.	54	S	AC I	colon
52.	55	S	AC I	sigmoid colon
53.	44	S	BG 2	colon descendens, (+ 2 metachronous: Ca ovarii)
54.	63	S	AC I	colon transversum
55.	45	S	RBG 4	colon ascendens
56.	59	S	RBG 5	rectum

[a] microsatellite instability status: H (unstabile high), L (unstabile low), S (stable)

[b] AC I,II (Amsterdam criteria), BG 2,3,4 (Bethesda guidelines), RBG 1,2,4,5 (Revised Bethesda guidelines)

Table 3. LOH results in patients with MSI-L or MSS tumors

Numb.	*MSH2* region			Numb.	*MLH1* region		
of pat.	D2S391	D2S123	D2S278	of pat.	D3S1561	D3S1611	D3S3685
3	+	+	+	1	+	+	+
1	U	+	+	1	+	U	+
1	+	+	-	1	U	+	U
1	-	-	+	2	+	U	U
				1	U	U	+
				2	U/-	-	+

+, LOH presence, -, LOH absence, U, data are uninformative because the patient is homozygote, uninformative heterozygote or MSI positive in analyzed marker

Table 4. Results of genetic and epigenetic characters in MSI-H tumors

Pat.	LOH[a]		Absence of protein expression [b]				*MLH1* promoter methylation[d]		Germline
code	*MSH2*	*MLH1*	MSH2	MLH1	PMS2	MSH6	region A	region B	mutation[f]
1.	U	U	-	+			+[e]	-	*MLH1*[h]
2.	U	U	-	+	+	-	+[e]	-	*MLH1*[h]
3.	U	-	+	-	-	+	+	+[e]	*MSH2*[h]
4.	U	-	+	-	-	+	+	-	both wt
5.	U	-	-	+	+	-	+[e]	-	both wt
6.	U	U	-	-			+[e]	-	*MLH1*[h]
7.	U	+	-	+	+	-	+[e]	+[e]	
8.	U	U	+	-			-	-	*MSH2*[h]
9.	U	-	+	-	-	+	+	-	both wt
10.	U	+	-	+			n.d.	n.d.	*MLH1*[h]
11.	U	U	-	+	+	-	+[e]	-	*MLH1*[g]
12.	U	U	-	-	-		+[e]	+[e]	both wt
13.	U	+	-	+	+	-	-	-	*MLH1*[g]
14.	-	U	-	+	+	-	+[e]	-	*MLH1*[g]
15.	U	U	-	+	+		+	-	*MLH1*
16.	U	+	-	+	+	-	+[e]	-	*MLH1*[g]
17.	U	-	n.d.	n.d.	n.d.	n.d.	+[e]	-	both wt
18.	U	+	-	+	+	-	-	-	*MLH1*[g]
19.	-	U		+[c]			+[e]	-	*MLH1*[g]
20.	U	U		+[c]	+[c]		+	-	*MLH1*[g]
21.	U	U		+[c]	+[c]		-	-	*MLH1*[g]
22.	U	U	n.d.	n.d.	n.d.	n.d.	+	-	*MSH2*[g]
23.	U	+	n.d.	n.d.	n.d.	n.d.	+	-	

[a] loss of heterozygosity in *MSH2* gene region (triplex D2S391, D2S123, D2S378) or in *MLH1* gene region (triplex D3S1561, D3S1611, D3S3685), U, data are uninformative because the patient is homozygote, uninformative heterozygote or MSI positive in all three analyzed markers, [b] absences of protein expressions were analyzed by immunostaining (IHC), [c] in normal tissue the protein expression was decreased, n.d., analyses can not be detected for quality of paraffin blocks (protein expression) and for DNA sample degradation (promoter methylation), [d] *MLH1* promoter methylation was analyzed by MSP, [e] PCR products of methylated DNA were detected equally in normal and tumor samples, [f] *MLH1* and *MSH2* genes were analyzed for germline mutation identification by sequencing, [g, h] characters of germline mutations were published in recent studies [47, 48].

produce unstable, truncated proteins. Samples with lacking MSH2 or MLH1 proteins showed simultaneously degraded MSH6 or PMS2 proteins as a result of alterations in binding sites of MSH2 or MLH1 proteins. We saw loss of the MLH1 protein in tumor tissues of 14 patients (70%); in 12 affected individuals, the results correlated with identified germline mutations. Lack of MSH2 protein staining was observed in four MSI-H persons (20%), two of those cases showed associations with *MSH2* germline mutation. The correlations of MMR protein expressions with germline mutations are presented in Table 4. Additionally, one patient with MSI-L tumor (code 26) manifested three unstable mono- and one dinucleotide markers and MSH6 protein was absent, suggesting a mutation in the MSH6 gene.

The methylation status of MSI-H cancers was evaluated in 22 patients, in both regions of *MLH1* promoter (Table 4). Out of 18 tumor samples manifesting the methylation of region A with no relation to the loss of MSH2 or MLH1 proteins, only in seven cases did the methylation phenotype occur preferentially in tumor tissue, relative to matched normal samples. Three persons presented CpG methylation of both regions equally in normal and tumor tissues; one of them is the car-

rier of *MSH2* germline mutation, in the second patient, no inherited mutation was determined by sequencing and in the third case, the inactivation of both alleles of *MLH1* gene by LOH and promoter hypermethylation was found. No methylation in any region was observed in five healthy young persons. Results from several methylation analyses are shown in Figure 2.

Pathogenic germline mutations were identified in 16 of 21 patients who were screened by direct genomic sequencing of the MMR genes. In these 16 cases, thirteen and three contained alterations in the *MLH1* and *MSH2* genes, respectively. Most of the mutations were described previously [47, 48]. The loss of immunostaining of relevant proteins was observed in 14 cases with identified mutations. Normal MLH1 protein expression was present only in the *MLH1* missense mutation carrier (code 6). A mutation in the *MSH2* gene was detected in patient No. 22 where IHC assay has not been performed due to unavailability of samples. None of the 23 patients with MSI-L or MSS phenotype was a carrier of the del616 mutation in the *MLH1* gene.

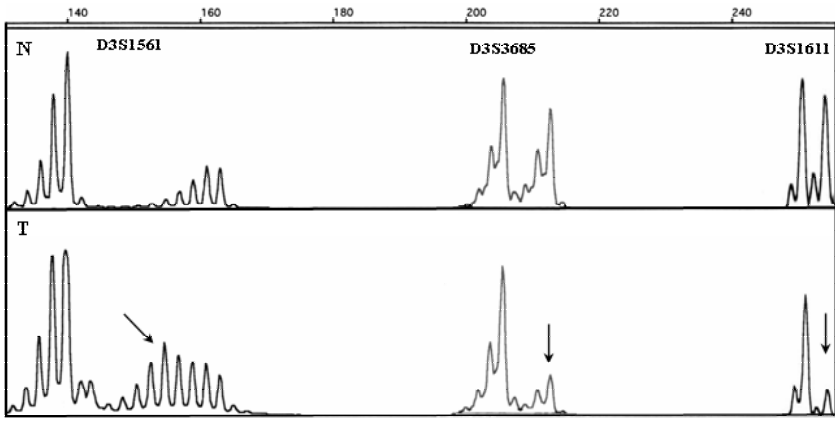

Figure 1. LOH analyse of markers in *MLH1* region. LOH was identified in markers D3S3685 and D3S1611 as reduced pattern of one allele in tumor samples in contrast to the matched normal DNA. The values of LOH were 3.4 and 4.3 for D3S3685 and D3S1611 markers, respectively. Marker D3S1561 manifested MSI in tumor DNA; therefore, it was uninformative for LOH evaluation. N, normal DNA; T, tumor DNA.

Discussion

CRC is one of the most common malignancies in the human population. The last European Union Report of Cancer showed that in the year 2000, population in Slovakia mani-

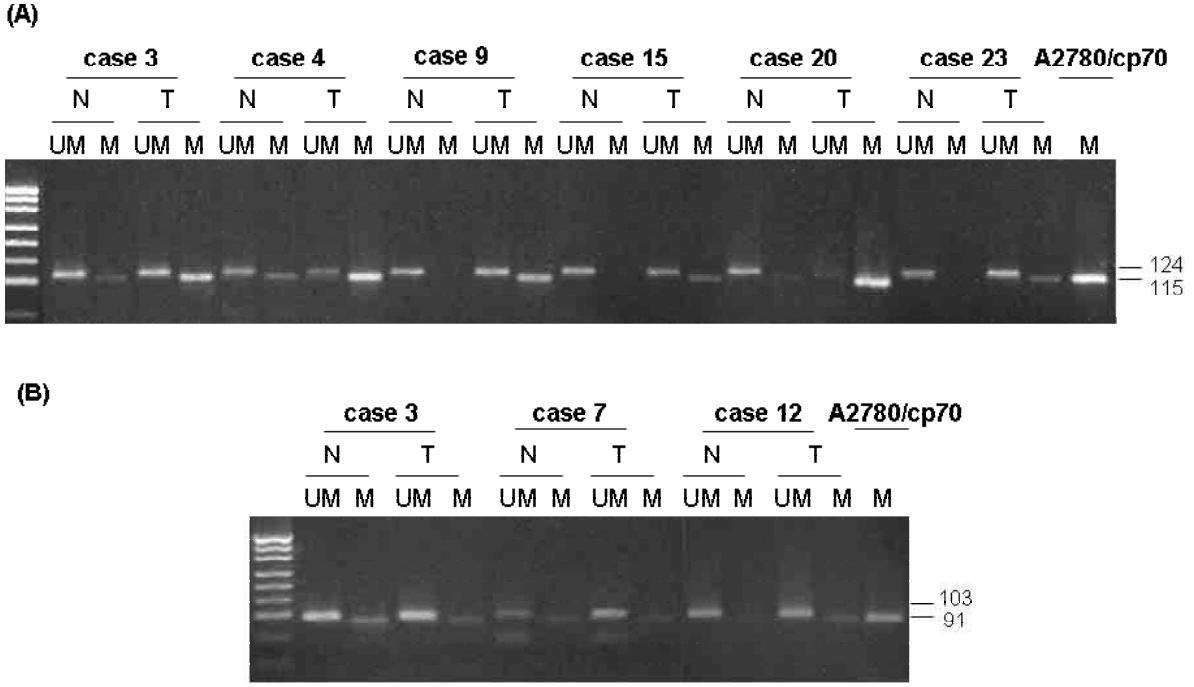

Figure 2. Methylation of *MLH1* promoter evaluated by MSP. (A) In region A of *MLH1* promoter in cases 9, 15, 20 and 23 methylated allele was shown only in tumor samples, the other patients manifested methylation in both normal and tumor samples. (B) Region B was slightly methylated in both normal and tumor samples and case 3 represents one of three patients with both methylated regions of MLH1 promoter. Completely methylated ovarian carcinoma cell line A2780/cp70 was used as positive control. N, normal DNA; T, tumor DNA; UM, PCR with specific primers for unmethylated substrate; M, PCR with specific primers for methylated substrate.

fested the fourth highest incidence of CRC, namely 52.65 cases per 100.000 inhabitants [49]. According to data of National Cancer Registry of Slovakia, 2852 new CRC patients were diagnosed in the same year. High mortality can be reduced by intensive prevention and medical surveillance in the global population and for high-risk persons, including Lynch-syndrome-suspected patients, a high-target program for early diagnosis and therapy must be administered.

The strict Amsterdam criteria are considered a reliable clinical indicator for identifying Lynch-syndrome patients, but random clustering of sporadic colorectal cancers can mimic this disease. In this study, we showed 24 patients fulfilling AC I or AC II criteria regardless MSI status. Six persons (25%) of them had MSS tumors, whereas their relatives presented carcinomas mostly in distal colon at advanced age, which is indicative of the sporadic origin of tumor. A similar frequency of sporadic MSS carcinomas in Amsterdam-positive families (17-25%) was observed in three independent studies [50, 15-16]. The introduction of Bethesda Guidelines broadened the Lynch-syndrome tumor range by accepting of gastric cancers. High-risk families in European region presented these carcinomas very rarely in contrast with members of Lynch-syndrome families in Asia, where gastric cancers are frequent [51]. In our study, families of four patients with MSI-H tumors (16%) had at least one person with stomach carcinoma. Consequently, three families evaluated by Bethesda Guidelines, included affected persons in two generations.

The predilection for right-sided colon is characteristic for more than 60% tumors in Lynch syndrome, but also for 84-94% of sporadic MSI-H cancers [52, 53], therefore its diagnostic value has been declined. We found proximal location of CRCs in 56% of unstable cases, where there was a positive family history, which predominantly predicted hereditary origin of the tumors. However, in early-onset unstable CRC patients, without any affected relatives, the right-sided location can indicate equally sporadic or inherited cause of cancer. On the other hand, authentic Lynch-syndrome families could be excluded from germline mutation screening for an inaccurate selection of index patient from multiple cases in family. A recent study documented that in kindred fulfilling Bethesda Guidelines, the patients with rectal carcinomas rarely manifested MSI phenotype. Most of them were sporadic cases in families suffered from Lynch syndrome in spite of relatively young age of diagnosis [28]. It is possible that some of the CRC families in our study have this syndrome, in spite of rectosigmoid location and MSS finding in evaluated patient. We were unable to test this hypothesis because the tumor tissues from other relatives were not available.

LOH analyses and evaluation of protein absence by IHC are effective pre-screening procedures for determining of damaged MMR gene, which is causal in Lynch-syndrome pathogenesis. The application of MSI markers in LOH analyses is economical, because it can be performed in one assay with MSI testing. On the other hand, in MSI-H tumors, many markers were unstable, resulting in uninformative LOH evaluation, which was partially compensated for by introducing of triple set of markers covering the *MLH1* or *MSH2* chromosome regions. The absence of MLH1 or MSH2 expression in our MSI-H group of patients (90%) was similar to previous studies (60-92%) [50, 15-16]. These findings accelerated the identification of germline mutations by DNA sequencing in most cases. In several patients with negative sequencing results, larger genomic alterations are very probable; therefore, the MLPA (Multiplex Ligation-dependent Probe Amplification) will be used for further testing.

A recent study demonstrated that the MSI-L phenotype is a genuine feature of several cancer types including colorectal, endometrial, or ovarian carcinomas, and is not a result of experimental errors [54]. Generally, that MSI-L CRC developed by distinct carcinogenetic pathway, independent of an MMR defect, has been suggested (for a review, see ref. [55]). Thus there is low probability to identify MMR germline mutations in patients without a family history of cancer, who suffered from MSI-L tumor.

The role of *MLH1* epigenetic silencing in Lynch-syndrome pathogenesis has not been sufficiently explained yet. In this study, the variable level of *MLH1* promoter hypermethylation, predominantly in distal region, was observed in most cases. Three tumors contained methylated sites in both regions, but only in one case,did the results correspond with the loss of MLH1 protein. In this patient, the germline mutation has not been identified yet and one allele was inactivated by LOH, similarly as in single patient reported previously [56]. In both studies, the vertical transmission of methylated allele was not verified due to a lack of availability of samples from other kindred. Thus, the hereditary origin of such epigenetic event remains controversial. In addition, our patient described above had two relatives with colon cancer at advanced ages that indicate the possibility of random clustering of sporadic cancers. In the second case presenting *MLH1* promoter methylation, only the absence of MSH2 protein staining was found; therefore, the crucial cause of MMR defect seems to be a defined germline mutation in *MSH2* gene. The role of methylated *MLH1* promoter in MMR defect was not explained even in the third patient, in whom there was normal expression of MMR proteins, and no germline mutations were found.

The *MLH1* promoter methylation in serum of CRC patients with MSI phenotype, who have distal or modal metastases [26], indicated contamination by tumor cells. However, multiple varied sites of methylation at the *MLH1* promoter were observed in normal DNA samples from patients with sporadic unstable CRCs, but the methylation was less dense compared to corresponding tumor tissues (20% vs. 80%) or in the hemiallelic state [57, 30]. No methylation was seen in DNA from blood samples of Lynch-syndrome patients [30], in contrast to our study, where *MLH1* methylation in both peripheral blood and normal mucosa, in addition to tumor tissues, was observed in most Lynch-syndrome patients, regardless of the presence of germline mutations in *MLH1* or *MSH2* genes. Given our data in combination with no methy-

lation findings in healthy young persons, we conclude that DNA hypermethylation is associated with tumorigenesis. Nevertheless, the epigenetic silencing of *MLH1* gene in hereditary tumors was not confirmed.

It is necessary to remark that, because the causal sequence for gene silencing has yet not been identified, responsible analysis of methylation status must include more than a ten CpG sites. In addition, *MLH1* gene expression is likely to be attenuated by the density of promoter methylation in regions up to -500 bp from transcription start [57]. For that reason, the examination of all CpG sites in the promoter sequence, predominantly in proximal region, is necessary.

Several clinical characteristics of Lynch-syndrome tumors have been previously observed in sporadic CRCs. Therefore, suspected patients should be examined by complex of molecular assays for reliable diagnosis. Epigenetic events seem to have no significant role in MMR deficiency of hereditary colorectal carcinomas, and differences in methylation status between sporadic and hereditary CRCs will be subject of the future study.

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References

[1] WATSON P, RILEY B. The tumor spectrum in the Lynch syndrome. Familial Cancer 2005; 4: 245–248.

[2] PELTOMAKI P, VASEN HF. Mutation associated with HNPCC predisposition — Update of ICG-HNPCC/INSiGHT mutation database. Dis Markers 2004; 20: 269–276.

[3] AARNIO M, SANKILA R, PUKKALA E et al. Cancer risk in mutation carriers of DNA-mismatch-repair genes. Int J Cancer 1999; 81: 214–218.

[4] VASEN HF, MECKLIN JP, KHAN PM, LYNCH HT. The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC). Dis Colon Rectum 1991; 34: 424–425.

[5] VASEN HF, WATSON P, MECKLIN JP, LYNCH HT. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. Gastroenterology 1999; 116: 1453–1456.

[6] RODRIGUEZ-BIGAS MA, BOLAND CR, HAMILTON SR et al. A National Cancer Institute Workshop on Hereditary Nonpolyposis Colorectal Cancer Syndrome: meeting highlights and Bethesda guidelines. J Natl Cancer Inst 1997; 89: 1758–1762.

[7] UMAR A, BOLAND CR, TERDIMAN JP et al. Revised Bethesda guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst 2004; 96: 261–268.

[8] PELTOMAKI P, VASEN HF. Mutation predisposing to hereditary nonpolyposis colorectal cancer: database and results of a collaborative study. The International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer. Gastroenterology 1997; 113: 1146–1158.

[9] HEMMINKI A, PELTOMAKI P, MECKLIN JP, et al. Loss of wild type MLH1 gene is a feature of hereditary nonpolyposis colorectal cancer. Nat Genet 1994; 8: 405–410.

[10] TOMLINSON IP, ILYAS M, BODMER WF. Allele loss occurs frequently at hMLH1, but rarely at hMSH2, in sporadic colorectal cancer with microsatellite instability. Br J Cancer 1996; 74: 1514–1517.

[11] LU SL, AKIYAMA Y, NAGASAKI H, et al. Loss or somatic mutations of hMSH2 occur in hereditary nonpolyposis colorectal cancer with hMSH2 germline mutations. Jpn J Cancer Res 1996; 87: 279–287.

[12] REYES CM, ALLEN BA, TERDIMAN JP Comparison of selection strategies for genetic testing of patients with hereditary nonpolyposis colorectal carcinoma. Effectiveness and cost-effectiveness. Cancer 2002; 95: 1848–1856.

[13] RAMSEY SD, BURKE W, CLARKE L. An economic viewpoint on alternative strategies for identifying persons with hereditary nonpolyposis colorectal cancer. Genet Med 2003; 5: 353–363.

[14] DEBNIAK T, KURZAWSKI G, GORSKI G et al. Value of pedigree/clinical data, inmmunohistochemistry and microsatellite instability analyses in reducing the cost of determining hMLH1 and hMSH2 gene mutations in patients with colorectal cancer. Eur J Cancer 2000; 36: 49–54.

[15] HALVARSOON B, LINDBLOOM A, RAMBECH E et al. Microsatelie instability analysis and/or immunostaining for the diagnosis of hereditary nonpolyposis colorectal cancer? Virchows Arch 2004; 444: 135–141.

[16] CHRISTENSEN M, KATBALLE N, WIKMAN F et al. Antibody-based screening for hereditary nonpolyposis colorectal carcinoma compared with microsatellite instabilite analysis and sequencing. Cancer 2002; 95: 2422–2430.

[17] HAMPEL M, FRANKEL WL, MARTIN E et al. Screening for the Lynch syndrome (hereditary nonpolyposis colorectal cancer). N Engl J Med 2005; 352: 1851–1860.

[18] ENGEL C, FORBERG J, HOLINSKI-FEDER E et al. German HNPCC Consortium. Novel strategy for optimal sequential application of clinical criteria, immunohistochemistry and microsatellite analysis in the diagnosis of hereditary nonpolyposis colorectal cancer. Int J Cancer 2006; 118: 115–122.

[19] WAGNER A, BARROWS A, WIJNEN JT et al. Molecular analysis of hereditary nonpolyposis colorectal cancer in the United States: high mutation detection rate among clinically selected families and characterization of an American founder genomic deletion of the MSH2 gene. Am J Hum Genet 2003; 72: 1088–1100.

[20] KANE MF, LODA M, GAIDA GM et al. Methylation of the hMLH1 promoter correlates with lack of expression of

hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Res 1997; 57: 808–811.

[21] CUNNINGHAM JM, CHRISTENSEN ER, TESTER DJ et al. Hypermethylation of the hMLH1 promoter in colon cancer with microsatellie instability. Cancer Res 1998; 58: 3455–3460.

[22] ESTELLER M, FRAGA MF, GUO M et al. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet 2001; 10: 3001–3007.

[23] POTOCNIK U, GLAVAC D, GOLOUGH R et al. Causes of microsatellite instability in colorectal tumors: implications for hereditary non-polyposis colorectal cancer screening. Cancer Genet Cytogenet 2001; 126: 85-96.

[24] HERMAN JG, UMAR A, POLYAK K et al. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA 1998; 95: 6870–6875.

[25] KIM HC, KIM CN, YU CS et al. Methylation of the hMLH1 and hMSH2 promoter in early-onset sporadic colorectal carcinomas with microsatellite instability. Int J Colorectal Dis 2003; 18: 196–202.

[26] GRADY WM, RAJPUT A, LUTTERBAUGH JD et al. Detection of aberrantly methylated hMLH1 promoter DNA in the serum of patients with microsatellite unstable colon cancer. Cancer Res 2001; 61: 900–902.

[27] BOLAND CR, THIBODEAU SN, HAMILTON SR et al. A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer, Cancer Res 1998; 58: 5248–5257.

[28] HOOGERBRUGGE N, WILLEMS R, VAN KRIEKEN HJ et al. Very low incidence of microsatellite instability in rectal cancers from families at risk for HNPCC. Clin Genet 2003; 63: 64–70.

[29] VEIGL ML, KASTURI L, OLECHNOWICZ J et al. Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. Proc Natl Acad Sci USA 1998; 95: 8698–8702.

[30] MIYAKURA Y, SUGANO K, AKASU T et al. Extensive but hemiallelic methylation of the hMLH1 promoter region in early-onset sporadic colon cancers with microsatellite instability. Clin Gastroenterol Hepatol 2004; 2: 147–156.

[31] RAEVAARA TE, VACCARO C ABDEL-RAHNAN WM et al. Pathogenicity of hereditary colorectal cancer mutation hMLH1 del616 linked of the functional protein. Gastroenterology 2003; 125: 501–509.

[32] BISGAARD ML, JAGER AC, MYRHOJ T et al. Hereditary non-polyposis colorectal cancer (HNPCC): phenotype-genotype correlation between patients with and without identified mutation. Hum Mutat 2002; 20: 20–27.

[33] MILLER SA, DYKES DD, POLESKY HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988; 16: 1215.

[34] FRIDRICHOVA I, ILENCIKOVA D, FRIEDL W et al. Approaches to identification of HNPCC suspected patients in Slovak population. Neoplasma 2000; 47: 219–226.

[35] DIETMAIER W, HARTMANN A, WALLINGER SE et al. Multiple mutation analyses in single tumor cells with improved whole genome amplification. Am J Pathol 1999; 154: 83–95.

[36] ODA S, OKI E, MAEHARA Y, SUGIMACHI K. Precise assessment of microsatellite instability using high resolution fluorescent microsatellite analysis. Nucleic Acids Res 1997; 25: 3415–3420.

[37] PARSONS R, MYEROFF LL, LIU B et al. Microsatellite instability and mutation of transforming growth factor b type II receptor gene in colorectal cancer. Cancer Res 1995; 55: 5548–5550.

[38] RAMPINO N, YAMAMOTO H, IONOV Y et al. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. Science 1997; 27: 967–969.

[39] National Center for Biotechnology Information. UniSTS: a comprehensive database of sequence tagged sites. Available at URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unists [Accessed 2006-07-24]

[40] SPIRIO L, JOSLYN G, NELSON L et al. CA repeat 30-70 kB downstream from the adenomatous polyposis coli (APC) gene. Nucleic Acid Res 1991; 19: 6348.

[41] WEBER JL, MAY PE. Dinucleotide repeat polymorphism at the D18S34 locus. Nucleic Acids Res 1990; 18: 6465.

[42] MAKELA TP, HELLSTEN E, VESA J et al. An Alu variable polyA repeat polymorphism upstream of L-myc at 1p32. Hum Mol Genet 1992; 1: 217.

[43] QIAGEN Multiplex PCR Handbook, p.21. Available at URL: http://www1.qiagen.com/literature/handbooks/PDF/PCRAndReverseTranscription/KitsAndEnzymes/PCR_Multiplex/1027945_HB_PCR_MP_0804_lr.pdf [Accessed 2006-07-24]

[44] GeneScan Reference Guide, ABI PRISM 310 Genetic Analyzer. Manual. Foster City, CA: Applied Biosystems USA; 2000.

[45] KLINGLER H, HEMMERLE C, BANNWART F et al. Expression of the hMSH6 mismatch-repair protein in colon cancer and HeLa cells. Swiss Med Wkly 2002; 132: 57–63.

[46] Protocol of CpGenomeTM Modification Kit. Rev. E:41422. Temecula CA: Chemicon International; Sept 2005. (Cat No S7820). Available at URL: http://www.chemicon.com/webfiles/PDF/S7820.pdf [Accessed 2006-07-24]

[47] ZAVODNA K, BUJALKOVA M, KRIVULCIK T et al. Novel and recurrent germline alterations in the MLH1 and MSH2 genes identified in hereditary nonpolyposis colorectal cancer patients in Slovakia. Neoplasma 2006; 53: 269–276.

[48] BARTOSOVA Z, FRIDRICHOVA I, BUJALKOVA M et al. Novel hMLH1 and hMSH2 germline mutations in the first HNPCC families identified in Slovakia. Hum Mutat 2003; 21: 449–453.

[49] CancerStats – Cancer in European Union, November 2004, Cancer Research UK, Available at URL: http://info.cancerresearchuk.org/cancerstats/geographic/cancerineu/ [Accessed 2006-07-24]

[50] WAHLBERG SS, SCHMEITS J, THOMAS G et al. Evaluation of microsatellite instability and immunochemistry for the prediction of germ-line MSH2 and MLH1 mutations in hereditary nonpolyposis colon cancer families. Cancer Res 2002; 62: 3485–3492.

[51] PARK YJ, SHIN KH, PARK JG. Risk of gastric cancer in hereditary nonpolyposis colorectal cancer in Korea. Clin Cancer Res 2000; 6: 2994–2998.

[52] KIM H, JEN J, VOGELSTEIN B et al. Clinical and pathological characteristics of sporadic colorectal carcinomas with DNA replication errors in microsatellite sequences. Am J Pathol 1994; 145: 148–156.

[53] YOUNG J, SIMS LA, BIDEN KG et al. Features of colorectal cancers with high-level microsatellite instability occurring in familial and sporadic settings. Am J Pathol 2001; 159: 2107–2116.

[54] HALFORD SER, SAWYER EJ, LAMBROS MB et al. MSI-low, a real phenomenon which varies in frequency among cancer types. J Pathol 2003; 201: 389–394.

[55] PAWLIK TM, RAUT CP, RODRIGUEZ-BIGAS MA. Colorectal carcinogenesis: MSI-H versus MSI-L. Dis markers 2004; 20: 199–206.

[56] GAZZOLI I, LODA M, GARBER J et al. A hereditary nonpolyposis colorectal carcinoma case associated with hypermethylation of the hMLH1 gene in normal tissue and loss of heterozygosity of the unmethylated allele in the resulting microsatellite instability – high tumor. Cancer Res 2002; 62: 3925–3928.

[57] FURUKAWA T, KONISHI F, MASUBUCHI S et al. Densely methylated MLH1 promoter correlates with decreased mRNA expression in sporadic colorectal cancer. Genes Chromosomes Cancer 2002; 35: 1–10.